Supplemental data to Woo et al. (2010)

Metabolite extraction using cold 50% (v/v) methanol. A culture sample of 1 ml was rapidly transferred into a 50 ml Falcon tube containing 5 g of ice precooled to -25°C and centrifuged for 2 min at 5311 x *g* and -20°C using a Sigma 4K15 centrifuge with four swing-out rotors (No.11150) (Sigma Labzentrifugen GmbH, Osterode am Harz, Germany) also precooled to -25°C. Ice and supernatant were immediately discarded, the cell pellet resuspended in 1 ml cold (0°C) 2.6% NaCl and then centrifuged again for 2 min at 5000 rpm and -20°C. The supernatant was discarded, 2 ml of 50% (v/v) methanol precooled to -30°C was added to the cell pellet and the tube vigorously mixed for 1 min. Then, samples were frozen in liquid nitrogen, thawed at room temperature and mixed for 1 min. The freeze-thaw cycle was repeated twice in order to achieve leakage of the cells and release of the intracellular metabolites. After the third thawing step, the cell extract was centrifuged for 10 min at 5311 x *g* and -4°C. 200 μL of the supernatant (cell-free extract) was transferred into a 0.5 ml Eppendorf tube and vaccum-dried overnight using a Vacufuge Centrifuge 5310 (Eppndorf AG, Hamburg, Germany) with fixed angle rotor in water-based mode. The extract was finally stored in a vacuum desiccator until it was used for GC-MS analysis.

Metabolite identification by GC-(EI/CI)-TOF-MS. The process used for the identification of "real" metabolites in the GC-MS spectra is schematically summarized in Fig. S1. It included the measurement of samples which differed only by the fact that the cultures were grown with either naturally labelled glucose or uniformly ^{13}C-C_6-labelled glucose. These samples were analysed both by GC-EI-MS and GC-CI-MS. The m/z shift of mass fragments owing to ^{13}C isotope incorporation allowed us to determine the number of carbon atoms in the metabolites. The application of the identification procedure shown in Fig. S1 resulted in three cases.

In case 1, "real" metabolites were identified as follows: Mass fragment patterns obtained by GC-EI-MS of the naturally labelled metabolites were used to search the NIST mass library for possible hits. Hits with a score above 700 were considered further. The unprotonated intact masses measured by GC-CI-MS of the naturally labelled sample were compared with the intact masses (including derivatization) calculated for the hits obtained by GC-EI-MS. "Real" hits should have the same mass in both cases. In addition, the number of carbon atoms obtained by subtraction of the unprotonated m/z

value obtained by GC-CI-MS of the ^{13}C-labelled sample from the unprotonated m/z value obtained by GC-CI-MS of the naturally labelled sample has to match the number of carbon atoms prediced for the hit from the NIST mass library. As an example the identification of a peak with a retention time of 8.76 min is described. The m/z pattern (59, 73, 100, 114, 147, 188, 189, 190, 262, 263) obtained by GC-EI-MS yielded a high-score hit of 900 (L-alanine) after searching the NIST library with NIST MS Search Program. The m/z value (unprotonated) at 8.76 min elution time obtained by GC-CI-MS of the ^{12}C sample (305.1625) matched the hit predicted from the GC-EI-MS analysis. In agreement, the number of three carbon atoms determined as described above matched with the predicted compound ($C_3H_7O_2N$).

In case 2, putative metabolites identified usually in small chromatographic peaks, but also in some large peaks (hexamethydisiloxan and its derivatives), were excluded as artifacts based on one of the following reasons: (i) The mass fragment pattern search in the NIST mass library led to top 10 hits with a score below 700. (ii) No mass shift was observed when comparing the ^{12}C- and ^{13}C-labelled samples, both in GC-EI-MS and GC-CI-MS. (iii) The unprotonated m/z value for the intact mass obtained by GC-CI-MS of the ^{12}C sample did not match with the intact mass predicted for the hits obtained by GC-EI-MS and NIST library search. A particular exception represents phosphoric acid, which represents a “real” metabolite, although no mass shift can be observed in the comparison of the ^{12}C- and ^{13}C-labelled samples.

Case 3 includes metabolites that could not be identified by searching the NIST library but by a procedure based on the measurement of the exact protonated mass of the intact compound (accuracy within 5 mDa) by GC-CI-MS. The mass obtained form the ^{12}C sample was first analysed using the elemental composition tool provided by the MarkerLynx software (Waters) to obtain possible chemical formulas. The resulting hits were then processed by the Matlab script “Metabolite Composition Analyzer” (Fig. S2), which eliminates the masses caused by the chemical derivatizations and analyzes the remaining hits for the H/C ratio, the N, O, P and S ratio, and the number of C atoms obtained by the comparison of the ^{12}C and ^{13}C-labelled samples. The hits that “survived” these checks were used to search automatically in public databases (PubChem and KEGG compound DB) for metabolites with the same chemical composition. This procedure was successful for candidates whose derivatized m/z was below 400 (Fig. S3). Metabolite candidates with m/z above 400 still await identification.

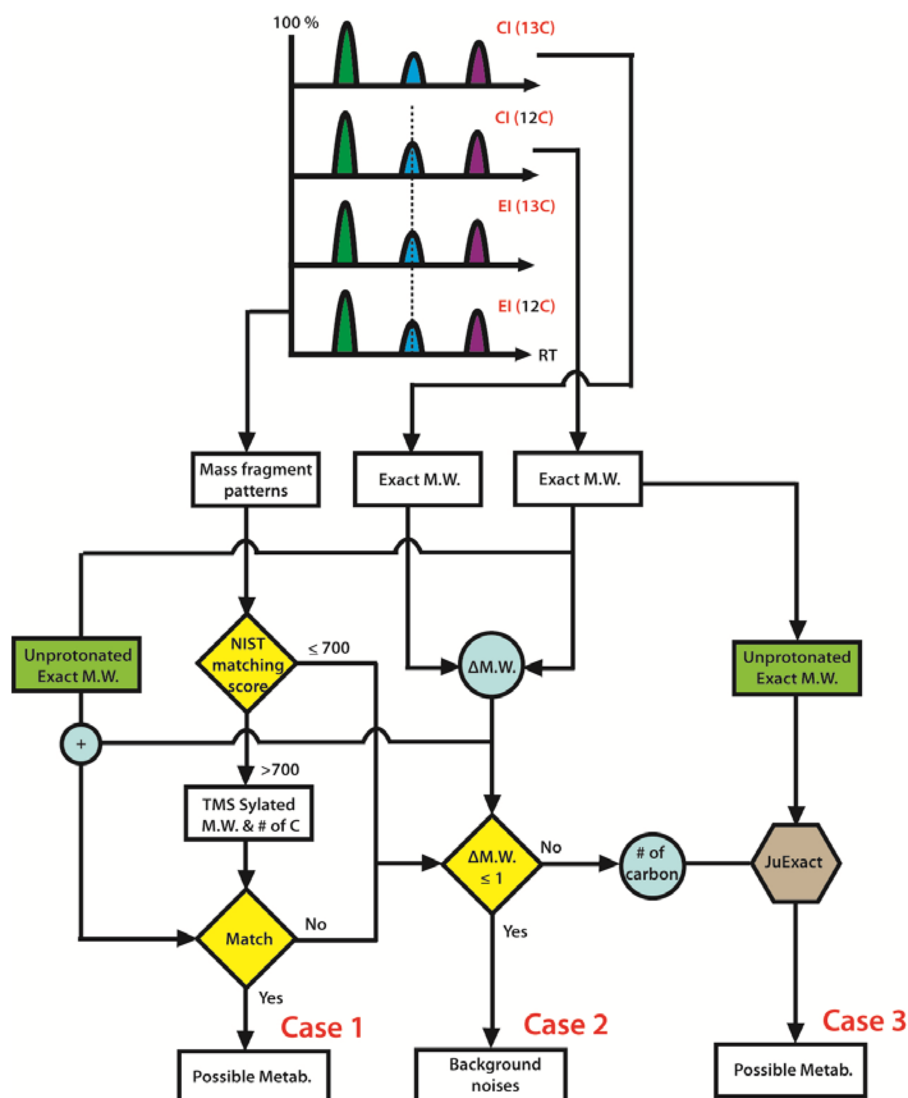

FIG. S1. Process to identify metabolites using GC-EI/CI-TOF mass spectrometry of ^{12}C-and ^{13}C-labelled cell extracts of *C. glutamicum*. For this purpose, cells were grown with either naturally labelled glucose (^{12}C sample) or $^{13}C_6$-labelled glucose (^{13}C sample). The extracted metabolites were analysed both by GC-EI-MS and GC-CI-MS. For details see text.

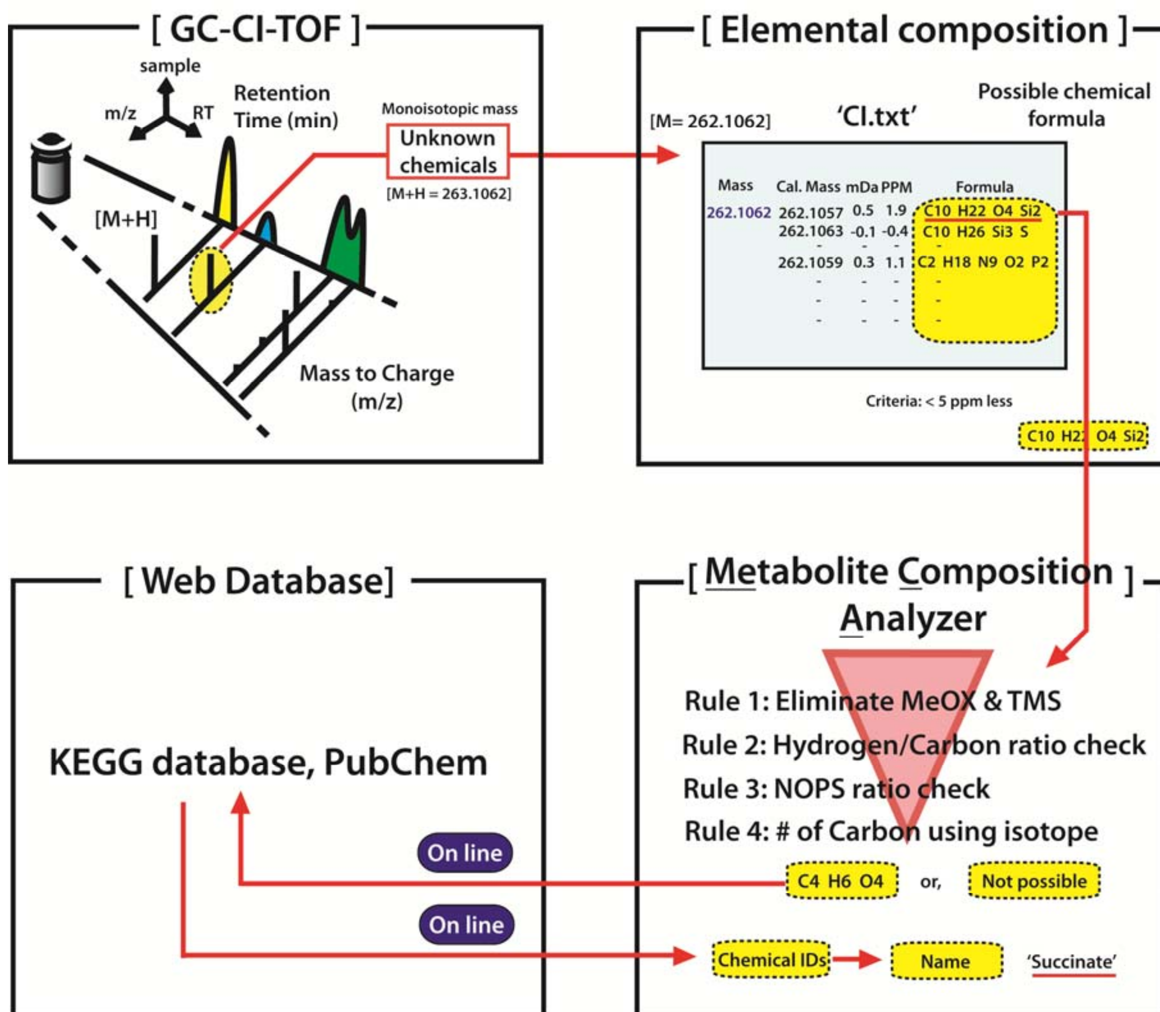

FIG. S2. A workflow to search metabolite candidates for unknown peaks and mass fragments using a Matlab script, called 'Metabolite Composition Analyzer' connected online to public chemical databases. In order to suggest metabolite candidates from unknown peaks, possible elemental compositions were calculated using the Elemental composition 4.0 tool provided by MassLynx software and the results were imported into a Metabolite Composition Analyzer tool that eliminates impossible candidates by applying a number of rules. The remaining candidates were used to search automatically in public databases (PubChem and KEGG compound DB) for metabolites with the same chemical composition. The results were shown as either text file or xml file.

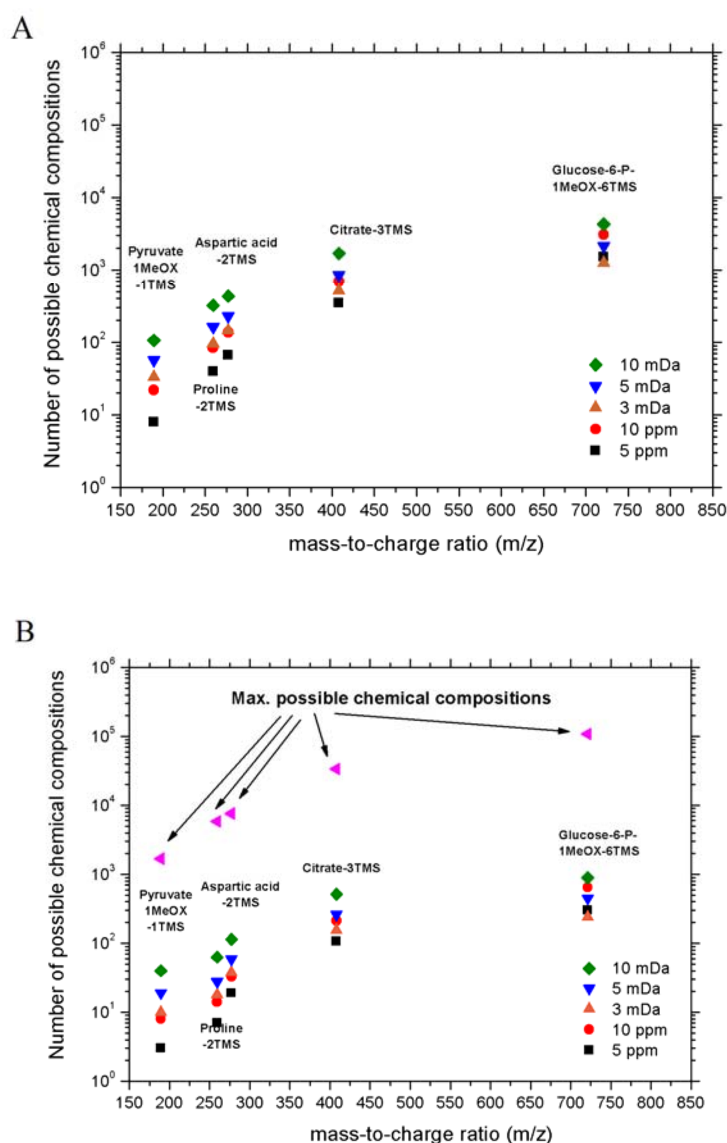

FIG. S3. Number of possible chemical compositions based on exact m/z measurement using only mathematical combination (A) or additionally chemical rules such as LEWIS, SENIOR, isotopic pattern, hydrogen/carbon ratios, and elemental ratios (B). Five different derivatized metabolites were used for calculation of the possible chemical compositions with different measurement errors (5 ppm, 10 ppm, or 3 mDa, 5 mDa, and 10 mDa), i. e. pyruvate-1MeOX-1TMS ($C_7H_{15}O_3NSi$, 189.0821 Da), proline-2TMS ($C_{11}H_{25}O_2NSi_2$, 259.1424 Da), aspartic acid-2TMS ($C_{13}H_{31}O_4NSi_3$, 349.1561 Da), citrate-3TMS ($C_{15}H_{32}O_7Si_3$, 408.1456 Da), and glucose 6-phosphate-1MeOX-6TMS ($C_{25}H_{64}O_9NPSi_6$, 721.2934 Da). In panel A, the mathematically possible combinations of the following chemical elements were calculated and displayed (n = number) that would result in the experimentally determined mass: C (n = 1–29), H (n = 1-101), O (n = 0-10), N (n = 0-5), S (n = 0-5), P (n = 0-5), Si (n = 1-11). In

panel B, possible chemical rules were applied in addition, including isotopic patterns (details given in the manual of MarkerLynx, Waters), to determine the maximally possible chemical compositions. For derivatized compounds with a mass >400 Da, the number of possible compositions is above 1000 for both variants. In these cases, additional rules besides the ones used in the Metabolite Composition Analyzer tool are required to eliminate false positives.

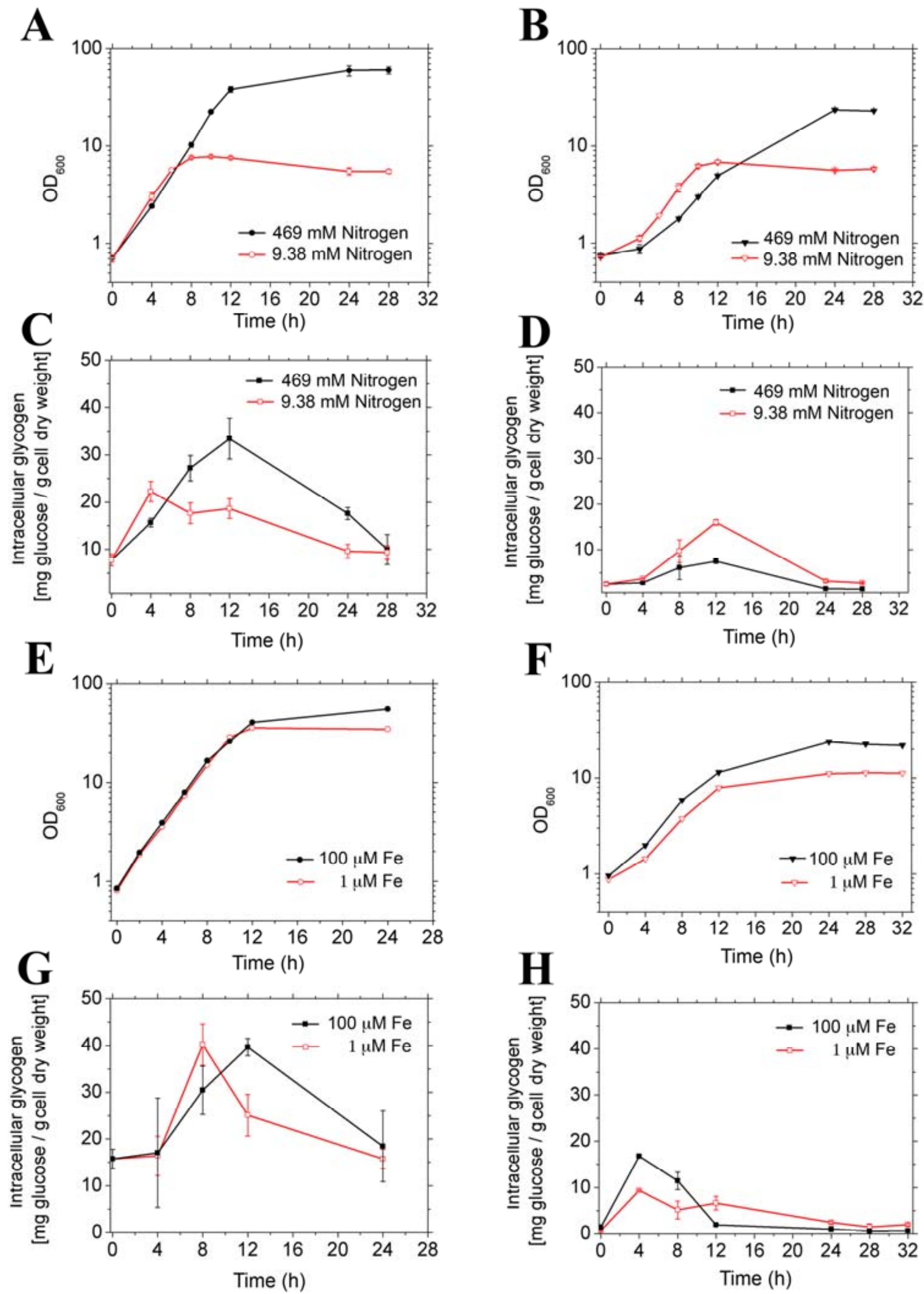

FIG. S4. Growth of and glycogen levels in *C. glutamicum* cultivated in CGXII minimal medium with either 222 mM glucose (left panels) or 300 mM potassium acetate (right panels). In panels A to D, the cells were grown under nitrogen excess and nitrogen limitation, wherease in panels E to H they were cultivated under iron excess and iron limitation. For nitrogen limitation, the concentrations of ammonium sulfate and urea were reduced to 1/50 of the original CGXII medium (20 g/l ammonium sulfate, 5 g/l urea). For iron excess and limitation, the medium contained either 100 µM $FeSO_4$ or 1 µM $FeSO_4$. The inoculum was precultivated twice under nitrogen or iron limitation.